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Physicochemical characteristics of glomerular basement membrane antigens in urine

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Biochemical analysis of glomerular basement membrane (GBM), first isolated by Krakower and Greenspoon [1], has shown that it is composed of collagen linked to glycoprotein [2]. In 1968, Hawkins and Cochrane reported that nephritic rabbits excreted into their urine material antigenically related to GBM [3]. Lerner and Dixon similarly found small amounts of it in the urine of normal rabbits [4], and McPhaul and Dixon detected it in normal human urine and showed it to be glycoprotein [5]. Urinary excretion of this GBM-related material, now generally called GBM antigens, is increased in various experimental [6–9] and human glomerular diseases [10–14]. Furthermore, physicochemically different GBM antigens have been observed in the urine of nephritic rabbits [3, 8, 9] and in pathologic human urine [14, 15].

We have used gel filtration and ion-exchange chromatographic procedures to fractionate and to characterize the GBM antigens present in normal and pathologic human urine. We were especially interested in studying the urinary GBM antigens of children with congenital nephrotic syndrome of Finnish type (CNF), because it has been suggested that synthesis of GBM is abnormal in this disease [16].

Methods

Patients. Twenty-four-hour urine samples were obtained from 7 children with renal disease; their clinical data are presented in Table 1. Pools of urine samples from 29 children with CNF, from 5 healthy children, and from laboratory staff without overt renal disease were also studied.

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Preparation of urine samples. Urine samples containing merthiolate (1:10,000) were filtered (Whatman 113V) and concentrated by vacuum dialysis with 8/32 Visking tubing (Scientific Instrument Centre Ltd., London). The 24-hour urine samples with a high protein content were concentrated 10 to 120 times, whereas normal urine samples were concentrated about 1,000 times. After concentration and dialysis (see below), the samples were centrifuged for 10 min at $\times 2000g$, filtered through 0.22-μm Millipore filter, and stored at $-20°$ C until analysis.

To obtain an enriched solution of GBM antigens, we fractionated the pooled CNF urine samples with ammonium sulphate. Solid ammonium sulphate was added gradually to concentrated urine with continuous stirring until 40% saturation was achieved. The precipitate was washed twice with 40% saturated ammonium sulphate solution, redissolved in 0.01 M phosphate buffered saline (PBS; pH, 7.3). No loss of GBM antigens during this procedure was detected by gel diffusion analysis.

Determination of protein concentration. The total protein content in the urinary samples of patients with heavy proteinuria was measured by the Biuret method. Albumin concentrations were determined by single radial immunodiffusion [17].

Anti-GBM serum. The isolation of human GBM and the preparation of anti-GBM sera have been described previously [12]. Briefly, GBM was separated by a modification of the method of Krakower and Greenspoon [1], sieving the renal cortex through metal screens, sonicating the sieved glomeruli, and separating GBM from cellular debris by centrifugation. The antiserum against human GBM was raised in rabbits and absorbed with glutaraldehyde-polymerized human plasma proteins [18, 19]. The immunoglobulin was concentrated about three

Table 1. Clinical details of the patients studied

Patient no.	Age	Sex	Diagnosis[a]	P_{Cr} *μmoles/liter*	BUN *mmoles/liter*	Urine protein *mg/hr/ m²*
1	2 wk	F	CNF	92	—	113
2	4 mo	F	DMS	—	13	610
3	3 yr	M	FGS	41	—	93
4	3 yr	F	SSNS	28	—	123
5	6 yr	M	SSNS, on steroids	36	—	58
6	6 yr	M	FGS	66	—	474
7	8 yr	F	AS	109	—	169

[a] Abbreviations are CNF, congenital nephrotic syndrome of Finnish type; DMS, diffuse mesangial sclerosis; FGS, focal glomerular sclerosis; SSNS, steroid-sensitive nephrotic syndrome; AS, Alport's syndrome.

times by precipitation with 50% saturated ammonium sulphate. An aliquot of anti-GBM serum was decomplemented by heating at 56° C, and absorbed with the buffy coat of a donor of a red cell type AB Rh-positive [20]. After absorption, no significant reduction in the titer of antibodies against GBM antigen was observed.

Detection of GBM antigens. GBM antigens were detected by double immunodiffusion against anti-GBM-serum using plates of 1.5% agar (Difco, Detroit, Michigan) in PBS (pH, 7.3). They were designated 1, 2, 3, and 4, as previously described [12]. The antigens in each sample investigated were identified by reactions of identity with a reference preparation of known antigen at high concentration. In selected samples, semiquantitative analysis was done by doubling dilution.

Immunoelectrophoresis. Immunoelectrophoresis was done in 1.5% agarose gel (Agarose B, Pharmacia Fine Chemicals, Uppsala, Sweden), in barbital buffer (pH, 8.6; I = 0.045). Reference preparations of whole human serum were included and developed with horse antihuman plasma protein serum (Wellcome, Beckenham, England).

Gel filtration. Serum and concentrated urine samples were dialyzed overnight against 0.1 M Tris-hydrochloric acid-0.2 M sodium chloride buffer (pH, 7.6) containing 0.02% sodium nitride, and fractionated on Sephadex G 200 (5 × 87 to 91 cm) with the same buffer. Sample volumes ranged from 6 to 25 ml. The columns were operated by upward flow at 54 to 61 ml/hr, and the effluent was monitored continuously by a Uvicord II (LKB Producter, Uppsala, Sweden) at 280 nm. Fifteen-minute fractions were collected, and the optical density (280 nm) of alternate fractions was determined with an Optica CF_4 spectrophotometer. Starting with the first protein peak, we made pools of eight consecutive fractions, and we concentrated them about 200 times for the detection of GBM antigens. The range of participation coefficient values (K_{av}) of each antigen was calculated according to Laurent and Killander [21], and the approximate molecular weights were estimated by the method of Andrews, with the albumin and IgG of the samples as internal standards [22].

Ion-exchange chromatography. Concentrated normal urine and ammonium sulphate precipitated CNF urine samples were dialyzed overnight against 0.01 M phosphate buffer (pH, 7.4) and fractionated on a column of Whatman DE-52 cellulose (3.2 × 39 cm) equilibrated with the same buffer and eluted with a gradient of increasing ionic strength from 0.01 to 0.2 M with a flow rate of 45 to 55 ml/hr. The volume of the applied samples was 30 ml (albumin content, 2.8 g/liter in normal urine and 1.1 g/liter in CNF urine). Fifteen-minute fractions were collected, and the optical density (280 nm) of the effluent was determined. The conductivities of alternate fractions were measured with a Philips PR 9500 conductivity meter, and corresponding molar concentrations of phosphate were read from a standard curve. Pools of eight individual fractions were made and concentrated 200-fold by vacuum dialysis prior to analysis.

Results

Three GBM antigens, Ag1, Ag2, and Ag3, were detected in concentrated pooled normal urine by immunodiffusion analysis. Of the three, Ag1 produced the most intense precipitation. After performing gel filtration of this concentrate, we identified a fourth antigen (Ag4), antigenically dissimilar to the other three, in the concentrated void volume material. Three antigens (Ag1, Ag2, and Ag3) were also detected in concentrated pooled CNF urine; but in contrast to normal urine, Ag3 gave the strongest precipitation. In the urine concentrates of individual patients, only one antigen, either Ag1 or Ag3, was detected, except for patient 2 (see Table

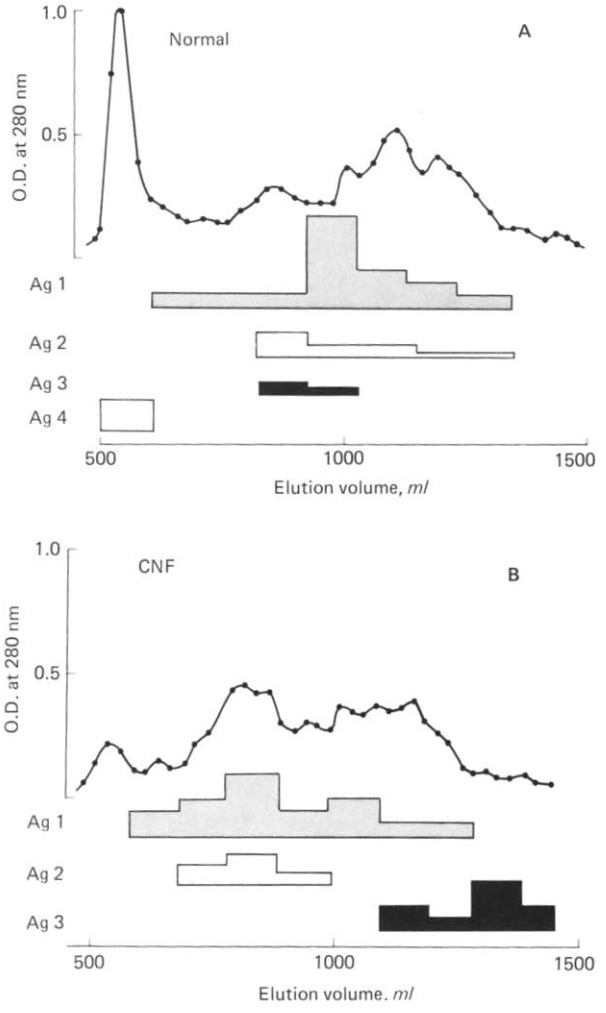

Fig. 1. *Sephadex G 200 gel filtration of concentrated pooled normal urine* **(A)** *and an ammonium sulphate-precipitated preparation of pooled urine from patients with congenital nephrotic syndrome of Finnish type (CNF)* **(B)**.

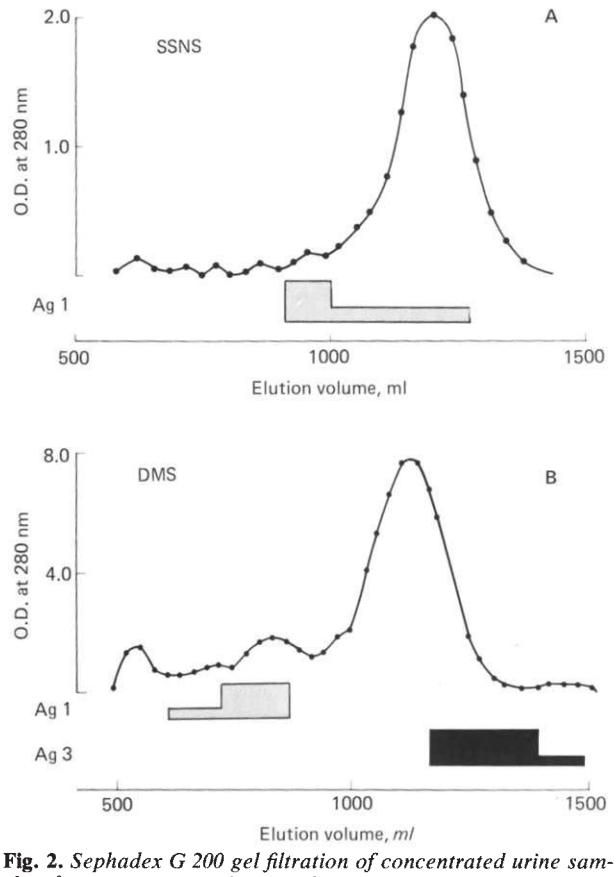

Fig. 2. *Sephadex G 200 gel filtration of concentrated urine samples from patients with steroid-sensitive nephrotic syndrome (SSNS)* **(A)** *and diffuse mesangial sclerosis (DMS)* **(B)**.

2) with diffuse mesangial sclerosis (DMS), in whose urine both antigens were detected. The identification of antigens in the fractions with relatively high antigen concentration was unambiguous but occasionally in some samples with low antigen concentration the demonstration of identity with a reference antigen was difficult, especially in the case of Ag3 in column fractions of normal urine.

Representative gel filtration profiles of normal, CNF, steroid-sensitive nephrotic syndrome (SSNS), and DMS urine concentrates and the semiquantitative estimates of GBM antigens in the pooled fractions of these separations are shown in Figs. 1 and 2.

K_{av} values and estimated molecular weights of the GBM antigens are given in Table 2. Ag1, especially in normal and pooled CNF urine, showed a wide range of molecular size, and in all samples Ag1 eluted with material having a molecular size of 60,000 to 200,000 daltons. In contrast, Ag3 of pathologic urine samples eluted over a smaller size range and had an average molecular weight less than that of albumin, but in normal urine it ranged from 90,000 to 180,000 daltons.

No GBM antigens were detected in unconcentrated normal serum or in the unfractionated serum of a bilaterally nephrectomized patient. After gel filtration of the latter, however, serum weakly precipitating Ag3 was detected in the third protein peak (K_{av}, 0.39 to 0.47; mol wt, 57,000 to 81,000 daltons).

Results from ion-exchange chromatography on the pooled normal and CNF urine samples are presented in Fig. 3. Ag1 was eluted with albumin at phosphate concentrations of 0.07 to 0.1 moles/liter, but some was eluted also at a phosphate concentration of 0.15 moles/liter. The Ag2 and a third antigen,

Table 2. Average participation coefficient (K_{av}) values and approximate molecular weights of urinary GBM antigens (Ag1, Ag2, Ag3, and Ag4)

Patient no.	Diagnosis[a]	Ag1		Ag2		Ag3		Ag4	
		K_{av}	Mol wt $\times 10^3$ daltons	K_{av}	Mol wt $\times 10^3$ daltons	K_{av}	Mol wt $\times 10^3$ daltons	K_{av}	Mol wt $\times 10^3$ daltons
1	CNF					0.37 to 0.53	56 to 90		
2	DMS	0.06 to 0.25	150 to 340			0.52 to 0.79	20 to 47		
3	FGS	0.17 to 0.34	100 to 210						
4	SSNS	0.05 to 0.58	35 to 350						
5	SSNS	0.26 to 0.54	45 to 150						
6	FGS	0.19 to 0.40	78 to 240						
7	AS					0.39 to 0.47	28 to 140		
Pooled normal urine		0.03 to 0.64	25 to 380	0.21 to 0.64	25 to 180	0.21 to 0.37	90 to 180	<0.03	>380
Pooled CNF urine		0.02 to 0.61	32 to 400	0.11 to 0.28	130 to 270	0.45 to 0.75	20 to 63		

[a] Abbreviations are defined in Table 1.

presumably Ag3, of normal urine were eluted from the column with the Ag1 peak. In contrast Ag3 and most of the Ag2 in the CNF urine were eluted with buffer of low molar concentration (see Fig. 3).

Two concentrated pools from the ion-exchange fractions of normal and CNF urine were analyzed by immunoelectrophoresis. Ag1 of both samples migrated anodically in the postalbumin area, whereas Ag3, detected only in CNF urine, migrated cathodically (Fig. 4).

Discussion

It has been shown clearly in several studies that urinary excretion of GBM antigens is increased in many types of glomerular disease [3, 6–14]. Opinions, however, differ on the number and physicochemical properties of these antigens. Usually, two or three distinct antigens have been detected in normal rabbit urine [4, 8, 9]. Hawkins and Cochrane observed that glomerular injury, induced in rabbits by injection of sheep antirabbit GBM-serum, was characterized by the excretion of antigens with a higher molecular weight than that of the GBM antigens in normal rabbit urine [3]. In similar circumstances, Boesken et al and Batsford and Hardwicke found a fourth GBM antigen as well, but some of these antigens were clearly smaller than those of normal rabbit urine [8, 9]. McPhaul and Dixon found, however, only two antigens in normal human urine [5].

By a combination of gel filtration and immunodiffusion, we have detected four distinct antigens in the urine of healthy people. Ag4 of the present study might be related to the antigen of McPhaul and Dixon which also eluted in the void volume of

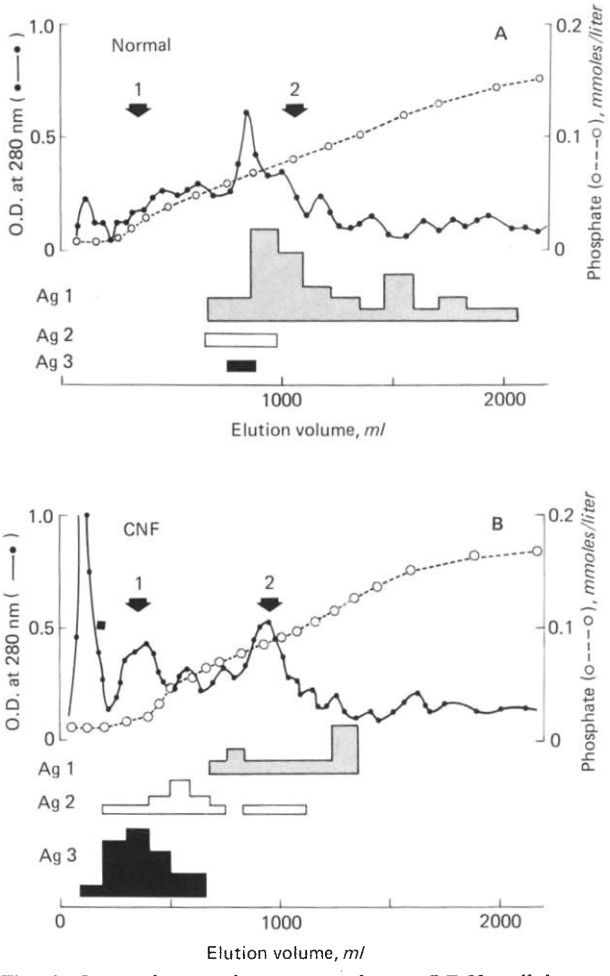

Fig. 3. *Ion-exchange chromatography on DE-52 cellulose of pooled normal urine* **(A)** *and an ammonium sulphate-precipitated preparation of pooled urine from patients with congenital nephrotic syndrome of Finnish type (CNF)* **(B).** The fractions labeled 1 and 2 were used in the immunoelectrophoretic analyses shown in Fig. 4.

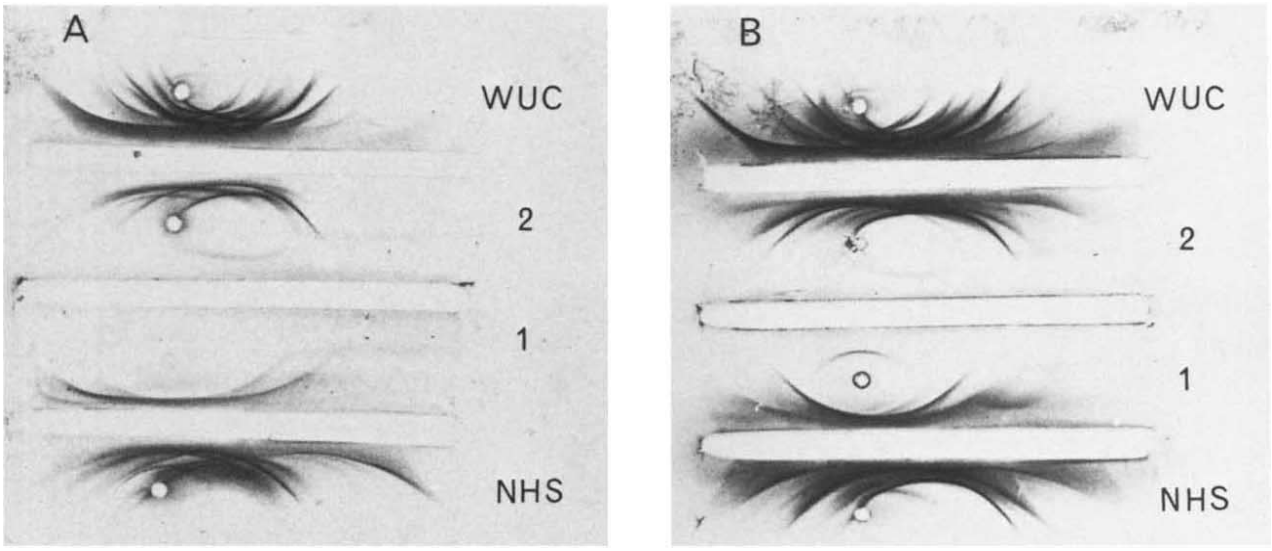

Fig. 4. A *Immunoelectrophoretic analysis of selected fractions (identified as 1 and 2 in Fig. 3 upper profile) from the ion-exchange chromatography of concentrated pooled normal urine.* **B** *Similar analysis of selected fractions from the ion-exchange chromatography of ammonium sulphate-precipitated pooled urine from patients with congenital nephrotic syndrome of Finnish type (identified as 1 and 2 in Fig. 3 lower profile).* In both A and B, the uppermost well contained unfractionated pooled normal urine concentrate (WUC), and the lower well contained normal human serum (NHS). Anti-GBM serum was added to the middle troughs and polyvalent anti-NHS serum to the upper and lower troughs of each plate. The precipitation arc produced by the albumin in fraction 2 of normal urine is not seen because it is in antigen excess. Anode is to the right.

Sephadex G 200. Ag1 of the present study possibly corresponds to the antigen of lower molecular weight in McPhaul and Dixon's study. The number of urinary GBM antigens detected, however, is influenced by the degree of concentration of the urine samples studied. This may explain why four antigens were detected in normal urine but not in the less concentrated pathologic urine samples in our study or in the normal urine studied by McPhaul and Dixon [3]. Alternatively, qualitative differences in the antisera raised against human GBM in different species may account for the observed differences between our study and that of McPhaul and Dixon. In addition, GBM shares antigenic determinants with other basement membranes of the body [23, 24], and thus an anti-GBM serum obtained by immunization with crude GBM cannot distinguish between antigens originating from GBM and those released by other basement membranes in the body.

Antigen 3, a minor component of normal urine, was the predominant GBM antigen of patients with CNF, DMS, and Alport's syndrome. The excretion of this antigen, however, is not specific to these disorders, because it was also detected in small amounts in normal urine, but the average molecular weight of Ag3 in pathologic urine was about 40,000 daltons, compared to 90,000 to 180,000 daltons in normal urine, suggesting a qualitative difference. Ag1 and Ag2 in the pathologic urine samples were about the same size as that found in normal urine. The ion-exchange chromatography and immunoelectrophoretic analyses suggest that Ag2 and Ag3 of CNF urine are more positively charged than these antigens in normal urine, but Ag1 was similar in both cases.

GBM antigens have been detected in the serum of both healthy individuals and anephric patients [3]. Huttunen et al showed previously that the antigen in the serum of nephrectomized CNF patients gave a reaction of identity with Ag3 in the urine of other CNF patients [12]. In this study, we confirmed the presence of Ag3 in the serum of a nephrectomized patient with another renal disorder. These results suggest that Ag3 may also originate from other basement membranes in the body, and be cleared through diseased glomeruli. In this case, the increased excretion of Ag3 of lower molecular weight in CNF urine might result from decreased tubular reabsorption of filtered Ag3. On the other hand, the following observations argue against an exclusively extrarenal origin for Ag3: *(a)* Ag3 could not be detected in the urine of patients with SSNS or focal glomerular sclerosis (FGS), *(b)* the urinary excretion of Ag3 in CNF increased with falling GFR [25], and *(c)* increased amounts of Ag3 are seen in the urine of patients not associated with tubular atrophy [12]. It seems most probable that urinary GBM anti-

gens are mainly released from glomeruli although Ag3 may be cleared to some extent from plasma into urine.

We have previously reported that the excretion of both Ag1 and Ag3 is increased in various types of glomerulonephritis and CNF, but not in SSNS [12]. Our present results show that the molecular weight of the antigen in the urine of patients with SSNS or FGS is similar to that of normal urine but different in the urine of patients with DMS, Alport's syndrome, and CNF. If urinary GBM antigens are membrane fragments, the quantitative and qualitative patterns in various diseases may reflect different mechanisms of GBM injury. Furthermore, the unusual GBM antigens in the urine of CNF patients may indicate an inherited disturbance of synthesis or degradation of GBM in this disorder.

Summary

Glomerular basement membrane (GBM) antigens in pooled normal urine and the pooled urine of infants with congenital nephrotic syndrome of Finnish type (CNF) have been characterized by gel filtration, ion-exchange chromatography, and immunodiffusion or immunoelectrophoretic analysis. Gel filtration studies were also done on urine samples from 7 individual children with various renal diseases. Using a single antiserum, we found four GBM antigens (Ag 1, 2, 3, and 4) in normal urine. Ag1 consistently produced the strongest precipitation in immunodiffusion analyses and was shown to be heterogeneous with respect to both molecular size (average mol wt, 100,000 daltons) and charge. In contrast, Ag3 gave the most intense precipitation in immunodiffusion analyses of urine concentrates from patients with CNF, diffuse mesangial sclerosis, and Alport's syndrome. Ag3 in these urine concentrates had an average mol wt of 40,000 daltons, somewhat smaller than that of Ag3 in normal urine. Ag1 was the only antigen to be detected in the urine of patients with steroid-sensitive nephrotic syndrome or focal glomerular sclerosis. In ion-exchange chromatography, Ag2 and Ag3 of CNF urine eluted at lower ionic strength than did the same antigens in normal urine, suggesting qualitative as well as quantitative differences. The observed heterogeneity of GBM fragments in different diseases may reflect different underlying mechanisms responsible for GBM damage.

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